4/pats

DESCRIPTION

METHODS OF SCREENING FOR TRANSPORTER INHIBITORS

Technical Field

The present invention relates to methods for expressing transporters having transporter activity, where the methods comprise using transporter-encoding genes to express transporters on the envelope of budding viruses. In addition, the present invention relates to viruses that express transporters having transporter activity, methods that use these viruses for measuring the transporter activity, and methods of screening for substances that inhibit or promote the transporter activity of the transporters.

Background Art

Mammals must take in nutrients from outside the body, and many transporter proteins (transporters) are known to exist in mammalian cells. These transporters mainly act to transport substances essential to the maintenance of life (amino acids, sugars, and such) into cells. In the physiological environment, cells often have multiple transporters that transport the same substrate. In these cases, the individual contribution of transporters to cellular uptake can be estimated using kinetic analysis (calculation of Km, Vmax, and so on; e.g., Wright E.M., Am. J. Physiol. Renal Physiol., 2001, 280: F10-18). Thus, identification of transport substrates and kinetic analysis of transporters are extremely important for revealing their physiological function and their potential in drug delivery.

Currently, methods for analysing transporter function use the following resources as materials: (1) primary cultured cells and cell membrane vesicles (such as lung cells and brush border membrane vesicles) comprising transporters isolated from living bodies; (2) cell lines derived from transporter-comprising cancer cells and so on (such as Caco-2 cells); (3) mammalian cells introduced with transporter genes (such as LLC-PK1 cells and MDCK cells) and Xenopus oocytes; and (4) insect cell membranes (such as Sf9 cell membranes) in which transporters have been expressed using baculovirus

expression systems. Of these, mostly used are gene expression systems from mammalian cells and Xenopus oocyte cells. However, even in mammalian and Xenopus oocyte cells introduced with transporter genes, activities from endogenous transporters can be detected, thus elevating background levels (Kanai Y. *et al.*, J. Clin. Invest. 93: 397-404 (1994); Kekuda R. *et al.*, J. Biol. Chem. 271: 18657-18661 (1996); Kekuda R. *et al.*, Am. J. Physiol. 272: G1463-1472 (1997); Yabuuchi H. *et al.*, J. Pharmacol. Exp. Ther. 286: 1391-1396 (1998); Hatanaka T. *et al.*, J. Clin. Invest. 107: 1035-1043 (2001)). For this reason, in some types of transporters, there are reports that describe an activity ratio of only two between cells introduced with genes and those not introduced with genes (parent cell lines). Carrying out kinetic analysis can be problematic in such gene-introduced cells with a low activity ratio.

In Xenopus oocyte cells introduced with transporter genes, transporter activity can be measured using electrophysiological methods. In transporters driven by Na and H ions, and substrates having an electric charge at physiological pH, transporter activity can be detected by measuring the electrical current caused by substrate transport. However, measuring transport activity is difficult when there is no driving force and also when substrates are electrically neutral at physiological pH. Kinetic analysis is also difficult in cases where transporter activity is observed but only a weak current can be detected. In addition, since electrophysiological methods require specific equipment, they are not simple or convenient.

The activity and substrate specificity of transporters that transfer drugs into cells has been reported to influence the drug's bioavailability (for example, Ganapathy, Leibach, Curr. Biol 3: 695-701 (1991); Nakashima *et al.*, Biochem. Pharm. 33: 3345-3352 (1984); Friedman, Amidon, Pharm. Res. 6:1043-1047 (1989); Okano *et al.*, J. Biol. Chem. 261: 14130-14134 (1986); Muranushi *et al.*, Pharm. Res. 6: 308-312 (1989); Friedman, Amidon, J. Control. Res. 13: 141-146 (1990)). In recent years, research on factors that fluctuate *in vivo* pharmacokinetics has clarified that drug-metabolising enzymes as well as drug-transporters influence the function of drugs in the body. Known drug-transporters include p-glycoprotein (Annu. Rev. Biochem.

58: 137 (1989)), multidrug resistance protein (Science 258: 1650 (1992); Cancer Res. 55: 102 (1995)), lung resistance protein (Ann. Oncl. 7: 625 (1996); Int. J. Cancer 73: 1021 (1997)), and organic cation transporter (Proc. Natl. Acad. Sci. USA 91: 133 (1994); Molec. Pharmacol. 51: 913 (1997)). Analysis of SNPs is being carried out for these drug-transporters in the same way as for drug-metabolizing enzymes. Transporter SNPs that bring about functional changes have been recently found. These SNPs are receiving attention as one of the factors causing fluctuations between individuals (Ryu S. *et al.*, J. Biol. Chem. 275: 39617-39624 (2000); Tirona R.G. *et al.*, J. Biol. Chem. 276: 35669-35675 (2001)). Currently, functional analysis of transporter SNPs mainly uses mammalian cells introduced with genes. However, this is speculated to be problematic for accurately detecting functional changes caused by SNPs in substrates having a low activity ratio compared to parent cell lines.

Disclosure of the Invention

The present invention was made considering the above circumstances. An objective of the present invention is to provide methods for measuring the target transporter activity, which have a low background level and a high degree of sensitivity. In addition, another objective of the present invention is to provide methods of screening for substances that inhibit or promote the transport activity of transporters, using the above methods.

Since viruses have no fundamental need to self-reproduce, the present inventors speculated that there was no physiological value in taking up substances essential to maintenance of life. Thus, they focused on the assumption that endogenous transporters may not be expressed (or maybe expressed in extremely low amounts) on viral envelopes. The method for measuring transporter activity using budding baculoviruses that do not express endogenous transporters on their envelopes are thought to have a low background level, and to enable a highly sensitive measurement of target activity. Furthermore, by using such methods, functional changes due to transporter SNPs can be measured for a broader range of substrates, and may be applied to tailor-made therapies.

Specifically, the present invention provides:

[1] a method for expressing a transporter having transporter activity, wherein the method comprises culturing a host infected with a recombinant virus that comprises a gene encoding the transporter, and expressing the transporter on the envelope of a budding virus released from the host;

[2] the method of [1], wherein the virus is a baculovirus;

[3] the method of [1] or [2], wherein the transporter is of a non-viral origin;

[4] the method of any of [1] to [3], wherein the transporter is a peptide transporter or an organic anion transporter;

[5] the method of [4] wherein the transporter is PepT1, PepT2, or OATP-C;

[6] a virus that expresses a transporter having transporter activity;

[7] the virus of [6], wherein the transporter is of a non-viral origin;

[8] the virus of [7] wherein the virus is a baculovirus;

[9] the virus of any of [6] to [8] wherein the virus is a budding virus;

[10] the virus of any of [6] to [9] wherein the transporter is a peptide transporter or an organic anion transporter;

[11] the virus of [10] wherein the transporter is PepT1, PepT2, or OATP-C;

[12] a method for measuring the activity of a transporter, wherein the method comprises expressing the transporter on a viral envelope;

[13] the method of [12] wherein the virus is a budding baculovirus;

[14] the method of [12] or [13] wherein the transporter is a peptide transporter or an organic anion transporter;

[15] the method of [14] wherein the transporter is PepT1, PepT2, or OATP-C;

[16] a method of screening for a substance that inhibits or promotes transport activity of a transporter, wherein the method comprises the following steps:

(a) expressing the transporter on a viral envelope,

(b) contacting the transporter with a test substance, and

(c) selecting a substance that inhibits or promotes the transport activity;

[17] the method of [16] wherein the virus is a baculovirus;

[18] the method of [16] or [17] wherein the virus is a budding virus;

[19] the method of any of [16] to [18], wherein the transporter is of a non-viral origin;

[20] the method of any of [16] to [19], wherein the transporter is a peptide transporter or an organic anion transporter;

[21] the method of [20] wherein the transporter is PepT1, PepT2, or OATP-C;

[22] the method of any of [16] to [21], which comprises immobilizing the virus on a support;

[23] the method of [22] wherein the virus is immobilized on the support through an antibody against an envelope protein expressed on the viral envelope; and,

[24] the method of [22] wherein the virus is immobilized on the support through a biotin-streptavidin reaction by biotinylating a protein expressed on the viral envelope.

The present invention relates to methods for expressing transporters having transporter activity, which methods comprise culturing a host infected with a recombinant virus that comprises a gene coding for a transporter, and expressing the transporter on the envelope of a budding virus released from the host. Herein, examples of a "transporter" include peptide transporters, amino acid transporters, and sugar transporters. More specifically, transporters such as those listed in Table 1 can be given as examples.

Table 1

Transporter	Driving force/ transport type	Amino acids	Trans-membrane	ncbi	Reference
4F2hc	LAT regulatory factor	529	1	P08195	Proc. Natl. Acad. Sci. U.S.A. 84 (18), 6526-6530 (1987)
AE4	Cl/HCO exchange transport	945	14	AAK16733	Commun. 282 (5), 1103-1109 (2001)
ATB^{0}/ASCT2	Na/neutral amino acid cotransport	541	10	Q15758	J. Biol. Chem. 271 (31), 18657-18661 (1996)
ATB^{0+}	Na/neutral and basic amino acids cotransport	642	12	AAD49223	J. Biol. Chem. 274 (34), 23740-23745 (1999)
$BAT1/b^{0+}$AT	Facilitated diffusion (amino acid)	487	12	P82251	Nat. Genet. 23 (1), 52-57 (1999)
BCRP	ATP/primary active transport	655	6	AAC97367	Proc. Natl. Acad. Sci. U.S.A. 95 (26), 15665-15670 (1998)
BSEP	ATP/primary active transport	1321	12	AAC77455	Nat. Genet. 20 (3), 233-238 (1998)
BTR1	Cl/HCO exchange transport	891	14	AAK16734	Commun. 282 (5), 1103-1109 (2001)
CNT1	Na/nucleoside cotransport	649	13	NP_004204	Am. J. Physiol. 272 (2), C707-C714 (1997)
CNT2	Na/nucleoside cotransport	658	14	O43868	Am. J. Physiol. 273 (6 Pt 2), F1058-F1065 (1997)
CNT3	Na/nucleoside cotransport	691	13	NP_071410	J. Biol. Chem. 276 (4), 2914-2927 (2001)
DRA/CLD	Cl/HCO exchange transport	764		P40879	Proc. Natl. Acad. Sci. U.S.A. 90 (9), 4166-4170 (1993)
EAAC1	Na/acidic amino acid cotransport	525	12	NP_004161	Genomics 20 (2), 335-336 (1994)
ENT1	Facilitated diffusion (nucleoside)	456	14	NP_004946	Nat. Med. 3 (1), 89-93 (1997)
ENT2	Facilitated diffusion (nucleoside)	456	14	AAC39526	Biochem. J. 328 (Pt 3), 739-743 (1997)
FORT	Folic acid	591	12	P41440	Commun. 206 (2), 681-687 (1995)
GAT1	Na/GABA cotransport	599	12	NP_003033	FEBS Lett. 269 (1), 181-184 (1990)
GAT3	Na/GABA cotransport	632	12	P48066	Recept. Channels 2 (3), 207-213 (1994)
GLUT1	Facilitated diffusion (glucose)	492	12	NP_006507	Science 229 (4717), 941-945 (1985)

GLUT2	Facilitated diffusion (glucose)	524	12	NP_000331	Proc. Natl. Acad. Sci. U.S.A. 85 (15), 5434-5438 (1988)
GLUT3	Facilitated diffusion (glucose)	496	12	NP_008862	J. Biol. Chem. 263, 15245-15248 (1988)
GLUT4	Facilitated diffusion (glucose)	509	12	NP_001033	J. Biol. Chem. 264 (14), 7776-7779 (1989)
GLVR1/PiT-1	Na/Pi cotransport	679	10	NP_005406	Cell Growth Differ. 1 (3), 119-127 (1990)
GLVR2/PiT-2	Na/Pi cotransport	652	10	NP_006740	J. Virol. 65 (11), 6316-6319 (1991)
LAT1	Facilitated diffusion (amino acid)	507	12	JG0165	Commun. 255 (2), 283-288 (1999)
LRP	ATP/primary active transport	896		NP_059447	Nat. Med. 1 (6), 578-582 (1995)
MCT1	H/organic anion cotransport	500	12	NP_003042	Genomics 23 (2), 500-503 (1994)
MCT2	H/organic anion cotransport	478	12	O60669	J. Biol. Chem. 273 (44), 28959-28965 (1998)
MCT3	H/organic anion cotransport	465	12	O15427	Biochem. J. 329 (Pt 2), 321-328 (1998)
MCT4	H/organic anion cotransport	487	12	O15374	Biochem. J. 329 (Pt 2), 321-328 (1998)
MCT5	H/organic anion cotransport	505	12	O15375	Biochem. J. 329 (Pt 2), 321-328 (1998)
MCT6	H/organic anion cotransport	523	12	O15403	Biochem. J. 329 (Pt 2), 321-328 (1998)
MDR1	ATP/primary active transport	1279	12	AAB69423	Cell 47 (3), 381-389 (1986)
MDR3	ATP/primary active transport	1279	12	P21439	EMBO J. 6 (11), 3325-3331 (1987)
MRP1	ATP/primary active transport	1531	17	P33527	Science 258 (5088), 1650-1654 (1992)
MRP2	ATP/primary active transport	1545	17	Q92887	Cancer Res. 56 (18), 4124-4129 (1996)
MRP3	ATP/primary active transport	1527	17	NP_003777	Cancer Res. 57 (16), 3537-3547 (1997)
MRP4	ATP/primary active transport	1325	12	NP_005836	Cancer Res. 57 (16), 3537-3547 (1997)
MRP5	ATP/primary active transport	1437	12	O15440	Cancer Res. 57 (16), 3537-3547 (1997)
MRP6	ATP/primary active transport	1503	17	O95255	Cancer Res. 59 (1), 175-182 (1999)

MRP7	ATP/primary active transport	1492	17		Cancer Lett. 162 (2), 181-191 (2001)
NaPi-3B	Na/Pi cotransport	690	8	NP_006415	Commun. 258 (3), 578-582 (1999)
NaSi-1	Na/Si cotransport	595	13	NP_071889	Genomics 70 (3), 354-363 (2000)
NHE1	Na/H exchange transport	815	12	P19634	Cell 56 (2), 271-280 (1989)
NHE2	Na/H exchange transport	812	12	NP_003039	Am. J. Physiol. 40 (2), 383-390 (1999)
NHE3	Na/H exchange transport	834	12	NP_004165	Am. J. Physiol. 269 (1 Pt 1), C198-C206 (1995)
NPT1	Na/Pi cotransport	467	6-8	Q14916	Genomics 18 (2), 355-359 (1993)
NPT2/NaPi-3	Na/Pi cotransport	639	8	NP_003043	Proc. Natl. Acad. Sci. U.S.A. 90, 5979-5983 (1993)
Nramp2/DCT1	Na/Fe cotransport	568	12	P49281	Mol. Immunol. 34 (12-13), 839-842 (1997)
NTCP2/ASBT	Na/bile acid cotransport	348	7	NP000443	J. Biol. Chem. 270 (45), 27228-27234 (1995)
OAT1	Facilitated diffusion (organic anion)	550	12	NP_004781	Commun. 255 (2), 508-514 (1999)
OAT2	Facilitated diffusion (organic anion)	548	12	NP_006663	
OAT3	Facilitated diffusion (organic anion)	568	12	NP_004781	Commun. 255 (2), 508-514 (1999)
OAT4	Facilitated diffusion (organic anion)	550	12	AAK68155	J. Biol. Chem. 275 (6), 4507-4512 (2000)
OATP-A	Facilitated diffusion (organic anion)	670	12	NP_066580	Gastroenterology 109 (4), 1274-1282 (1995)
OATP-B	Facilitated diffusion (organic anion)	709	12	NP_009187	Commun. 273 (1), 251-260 (2000)
OATP-C	Facilitated diffusion (organic anion)	691	12	BAA78639	Commun. 273 (1), 251-260 (2000)
OATP-D	Facilitated diffusion (organic anion)	710	12	BAA89287	Commun. 273 (1), 251-260 (2000)
OATP-E	Facilitated diffusion (organic anion)	722	12	BAA89288	Commun. 273 (1), 251-260 (2000)
OCT1	Facilitated diffusion (organic cation)	554	12	NP_003048	Mol. Pharmacol. 51 (6), 913-921 (1997)
OCT2	Facilitated diffusion (organic cation)	555	12	NP_003049	DNA Cell Biol. 16 (7), 871-881 (1997)

OCT3	Facilitated diffusion (organic cation)	551	12	NP_035525	Genomics 55 (2), 209-218 (1999)
OCTN1	H/organic cation	551	11	NP_003050	FEBS Lett. 419 (1), 107-111 (1997)
OCTN2	Na/organic cation cotransport	557	12	O76082	Commun. 246 (3), 589-595 (1998)
PGT	Facilitated diffusion (organic anion)	643	12	NP_005612	Commun. 221 (2), 454-458 (1996)
rBAT	BAT1 regulatory factor	685	1	AAA81778	J. Biol. Chem. 268 (20), 14842-14849 (1993)
SDCT1/NaDC-1	Na/dicarboxylic acid cotransport	592	8	NP_003975	Am. J. Physiol. 270 (4 Pt 2), F642-F648 (1996)
SGLT1	Na/glucose cotransport	664	14	NP00334	Proc. Natl. Acad. Sci. U.S.A. 86 (15), 5748-5752 (1989)
SGLT2	Na/glucose cotransport	672	14	NP_003032	Am. J. Physiol. 263 (3 Pt 2), F459-F465 (1992)
SGLT3/SAAT1	Na/glucose cotransport	659	14	P31636	J. Biol. Chem. 268 (3), 1509-1512 (1993)
SLC26A6	Cl/HCO exchange transport	738	11	NP_075062	Genomics 70 (1), 102-112 (2000)
SVCT1	Na/vitamin C cotransport	598	12	NP_005838	Biochim. Biophys. Acta 1461 (1), 1-9 (1999)
UT2	Urea (Facilitated diffusion)	397	10	Q15849	FEBS Lett. 386 (2-3), 156-160 (1996)

Preferable transporters in the present invention are peptide transporters or organic anion transporters, and especially preferable are PepT1, Pept2, and OATP-C. The nucleotide and amino acid sequences of PepT1 and PepT2 are known (human PepT1: GenBank XM_007063, J. Biol. Chem. 270(12): 6456-6463 (1995); human PepT2: GenBank NP_066568, XM_002922, Biochem. Biophys. Acta. 1235:461-466 (1995); mouse PepT1 GenBank AF205540, Biochim. Biophys. Acta. 1492: 145-154 (2000); mouse PepT2: GenBank NM_021301, Biochim. Biophys. Res. Commun. 276: 734-741 (2000)). Furthermore, the nucleotide and amino acid sequence of OATP-C are also known (Table 1: Commun. 273(1), 251-260 (2000)). However, the transporters of the present invention are not particularly limited thereto, as long as they can be expressed on a viral envelope.

Genes encoding the transporters, for example, those listed in Table 1, are registered with the National Centre for Biotechnology

Information (NCBI) under the listed accession numbers. For example, based on this sequence information, cDNA libraries or genomic libraries can be screened to obtain genes coding for transporters. More specifically, for example, cDNA or genomic libraries are screened using probes (antibodies against target transporters, or oligonucleotides that hybridise to nucleotide sequences coding for target transporters). Screening can be carried out, for example, by following the standard methods described by Sambrook *et al.* in Chapters 10 to 12 of "Molecular Cloning: A Laboratory Manual" (New York, Cold Spring Harbor Laboratory Press, 1989). Alternatively, genes encoding target transporters can be isolated using PCR (see e.g., Chapter 14 in the above-mentioned Sambrook *et al.*, 1989).

As methods for expressing transporters on viral envelopes, for example, the method of WO98/46777 or Loisel *et al.* for expressing envelope proteins using budding baculoviruses can be used (Loisel, T.P. *et al.*, Nature Biotech. 15: 1300-1304 (1997)). More specifically, a recombinant vector for insect cells comprising a gene encoding a transporter is constructed, and inserted, along with baculoviral DNA, into insect cells such as Sf9. The transporter encoded by the recombinant vector is then expressed on mature viral particles (virions), which are released by infected cells to the outside of cells prior to infected cell death. Thus recombinant viruses that express the transporter can be obtained.

In the present invention, a budding virus is a virus that is released from infected cells by budding. Generally, viruses covered by an envelope can bud from cells infected with these viruses, even when the cells have not been destroyed, and are released continuously. On the other hand, adenoviruses that are not covered by an envelope, and herpes viruses that are covered by a nuclear envelope, are released from the cells all at once upon their destruction. In the present invention, budding viruses are particularly preferable. In addition, hosts infected with a recombinant virus in the present invention can be suitably selected by those skilled in the art, depending on the type of virus used, so long as viral replication is possible in the host. For example, insect Sf9 cells can be used when using baculoviruses. Generally, protein expression systems using baculoviruses and insect cells may be useful because modifications

such as fatty acid acetylation or glycosylation are carried out at the same time as translation or post-translation, in the same way as in mammalian cells. In addition, the expression level of heterologous proteins in such systems is greater than that in mammalian cell systems (Luckow V.A. and Summers M.D., Virol. 167: 56 (1988)).

The present invention also provides viruses that express transporters comprising transporter activity. Examples of these viruses include baculoviruses, papillomaviruses, polyomaviruses, simian virus 40 (SV40), adenoviruses, Epstein-Bar virus (EBV), and retroviruses. In the present invention, particularly preferable viruses include the AcMNPV (Invitrogen) baculovirus, and budding viruses. In addition, the transporters expressed by the viruses are preferably of a non-viral origin, for example the transporters in Table 1. Of these, peptide transporters and organic anion transporters are preferable, and Pept 1, PepT2, and OATP-C are even more preferable.

The viruses expressing transporters having transporter activity of the present invention can be obtained by, for example, culturing a host that has been infected with a recombinant virus comprising a gene that codes for a transporter. Alternatively, using methods such as the above-mentioned methods of WO98/46777 and Loisel *et al* (Loisel, T.P. *et al.*, Nature Biotech. 15: 1300-1304 (1997)), a recombinant vector encoding a transporter can be inserted into an insect cell along with a baculovirus, and transporters can be expressed on the envelope of the baculovirus which is released outside of the cell. In addition, using methods like that of Strehlow *et al.* (D. Strehlow *et al.*, Proc. Natl. Acad. Sci. USA. 97: 4209-4214 (2000)), packaging cells such as PA317 can be infected with recombinant Moloney murine leukemia viruses, which are constructed using vectors derived from Moloney viruses introduced with transporter-encoding genes, and the transporters can be expressed on the envelope of the viruses released outside of the cells. However, the viruses of the present invention that express transporters having transporter activity are not limited to those that are constructed using the above methods. They include viruses constructed using any method as long as transporters can be expressed in viral particles or on viral surfaces.

Recombinant viruses constructed as described above can be purified using known methods. For example, known methods for purifying viruses include: augmented density gradient centrifugation (Albrechtsen *et al.*, J. Virological Methods 28: 245-256 (1990); Hewish *et al.*, J. Virological Methods 7: 223-228 (1983)), size exclusion chromatography (Hjorth and Mereno-Lopez, J. Virological Methods 5: 151-158 (1982); Crooks *et al.*, J. Chrom. 502: 59-68 (1990); Mento S.J. (Viagene, Inc.) 1994 Williamsburg Bioprocessing Conference), affinity chromatography using monoclonal antibodies, sulphated fucose-containing polysaccharides and the like (Najayou *et al.*, J. Virological Methods 32: 67-77 (1991); Diaco *et al.*, J. Gen. Virol. 67: 345-351 (1986); Fowler, J. Virological Methods 11: 59-74 (1986); TOKUSAIHYOU No. 97/032010 (Unexamined Publication of Japanese National Phase Patent Application)), and DEAE ion exchange chromatography (Haruna *et al.*, Virology 13: 264-267 (1961)). Viruses that express transporters of the present invention are not limited to these, and can be purified using the above methods, or combinations thereof.

The present invention relates to methods for measuring the activity of transporters, which comprise expressing transporters on viral envelopes. For example, measurement of transporter activity using budding baculoviruses can be carried out by the following method. First, if necessary, a substrate to be taken into the virus by the transporters is labelled so as to be detected. For example, the substrate is labelled with radioactive substances, fluorescence, or so on. Next, the substrate is mixed with the budding baculovirus that expresses the transporter, and reacted at 37°C. After a set length of time, the reaction solution is transferred onto a filter such as a cellulose membrane. The substrate taken into the virus is separated by vacuum filtration from the substrate that was not taken up. The filter is washed several times using an ice-cold buffer, and the substrate concentration in the viruses which are trapped on the filter is determined using a liquid scintillation counter, a fluorescence detector, HPLC, or such. Nonspecific uptake can be detected by the substrate uptake into wild type viruses that do not express the transporter. In addition, nonspecific uptake can also be evaluated by carrying out experiments on substrate uptake by coexisting the

substrate with transporter inhibitors, or if the substrate is radioactive, by coexisting it with an excess of unlabelled substance. Non-specific uptake can be evaluated by carrying out uptake experiments at 4°C.

As an alternative method, budding baculovirus solutions expressing a transporter can be added to a 96-well plate and incubated overnight at 4°C to perform plate coating. Alternatively, antibodies against proteins such as gp64 protein, which is highly expressed on viral envelopes, can be added to a 96-well plate, and incubated overnight at 4°C. After this, budding baculoviruses that express the transporter are added to the plate. Antibodies against membrane proteins, such as anti-gp64 antibodies (Novagen, Clontech), can also be used to coat the plate with the viruses. A substrate is then added to the plate, and reaction begins. After a set time, the plate is washed with ice-cold buffer, and substrates that were not taken up by the viruses are removed. The amount of substrates taken up into the virus is measured using a liquid scintillation counter, fluorescence detector, HPLC, or so on. If non-specific adsorption is high, blocking can be carried out prior to measuring activity, using skim milk or such. Non-specific uptake can be detected by substrate uptake into wild-type viruses not expressing the transporter. In addition, transporter inhibitors can be coexisted with the substrate to detect non-specific uptake. Alternatively, when the substrate is a radioactive substance, non-specific uptake can also be evaluated by carrying out uptake experiments by coexisting the substrate with an excess of unlabeled substances. Furthermore, uptake experiments can be carried out at 4°C to evaluate non-specific uptake.

Usually, cell membrane vesicles prepared from biological resources, cultured cells, and such are preserved in a deep freezer or in liquid nitrogen. However, budding baculoviruses can be preserved at 4°C, and do not require any special freezing devices. In addition, there are no complicated steps such as cell culturing, and there is no requirement for special equipment when measuring activity, as used in electrophysiological methods. Thus, budding baculovirus expression systems are simple methods for measuring transporter activity.

The methods of the present invention for measuring the transporter activity that comprise expressing transporters on viral envelopes can also be applied in searching for substances that inhibit or promote the transporter activity. In particular, methods using budding baculovirus expression systems are simple, and useful in identifying substances that inhibit or promote the transporter activity. Specifically, the methods of the present inventions produce, for example, budding baculoviruses that express target transporters. The radioactive or fluorescent substrates of those transporters are mixed with test substances, and added to the transporter-expressing viruses. Before adding the substrates, compounds can be preloaded to the viruses. Transport activity in the absence of a test substrate is taken as 100, and substances that inhibit or promote the transporter activity are searched for by using changes in activity in the presence of the test substrate as an index. Whether or not the test compound is inhibiting or promoting the transporter activity can be judged by known methods, for example, by labeling the transport target substrate (e.g. peptides in the case of peptide transporters) with a radioactive substance (such as ^{14}C) or fluorescent substance, and then measuring the amount of that substrate that is taken up by a transporter-expressing virus, etc.

Examples of test substances in the methods of screening for substances that inhibit or promote transport activity of the transporters of the present invention include, but are not limited to, purified or crude proteins (comprising antibodies), gene library expression products, synthetic peptide libraries, cell extracts, cultured cell supernatants, products of fermentation microorganisms, marine organism extracts, vegetable extracts, synthetic low molecular weight compound libraries, peptides, non-peptide compounds, and natural compounds.

Transporters expressed on viral envelopes can be contacted with test compounds in the form of, for example, a purified protein, a form bound to a carrier, a fusion protein with another protein, or a membrane fraction. Herein, examples of carriers on which viruses can be immobilized include synthetic or natural organic high molecular weight compounds, inorganic materials such as glass beads, silica gel, alumina, and active carbon, and these materials coated with

polysaccharides or synthetic high molecular weight molecules. Examples of organic high molecular weight compounds comprise a large number of compounds, including polysaccharides such as agarose, cellulose, chitin, chitosan, sepharose, and dextran, polyesters, polyvinyl cholorides, polystyrenes, polysulfones, polyether sulphones, polypropylenes, polyvinyl alcohols, polyamides, silicon resins, fluorocarbon resins, polyurethanes, polyacrylamides, and derivatives thereof. However, so long as the viruses can be immobilized, it is understood that the compositions of the compounds are not especially limited. The form of the carrier is also not particularly limited, and examples include membranes such as a plate, fibers, granules, hollow filaments, nonwoven fabrics, porous forms, and honeycomb forms. However, in the present invention, simplicity of immobilization makes commercially available plates especially preferable. By changing the form, surface area and such of these carriers, the contact area of test compounds can be controlled. Viruses can be immobilized to carriers using, for example, antibodies against the envelope proteins expressed in the viruses. In addition, immobilization onto carriers can also be achieved using streptoavidin, avidin or such when biotinylated beforehand.

The physiological function of transporters can be elucidated by searching for inhibitors or promoters of transporter activity. At the same time, those inhibitors or promoters may be applied to developing pharmaceutical agents for diseases caused by transporter abnormalities.

The present invention's budding baculoviruses that express promoters, and the envelope portions that comprise a transporter of those viruses, can be used as screening antigens or immune antigens when producing transporter antibodies. Preparation of such an antigen can be carried out, for example, according to the methods using baculoviruses described in WO98/46777.

Conventionally, in the construction of anti-transporter antibodies, it was problematic to use an active transporter as an immunogen. However, transporters that are expressed by the methods of the present invention have been confirmed to have transporter activity. Thus, an active transporter can be used as an immunogen by using the present invention's transporter-expressing viruses, or

envelope portions that comprise a transporter of those viruses.

Therefore, it is extremely useful to construct antibodies using, as immunogens, the present invention's transporter-expressing viruses and envelope portions that comprise a transporter of those viruses.

Thus, the present invention provides methods for constructing anti-transporter antibodies, which methods comprise using, as immunogens, the present invention's transporter-expressing viruses or envelope portions that comprise a transporter of those viruses. The present invention also provides the antibodies constructed using these methods.

Transporter antibodies of the present invention can be constructed by those skilled in the art, using known methods where non-human animals are administered, by subcutaneous or intraperitoneal injection, several times with transporter-expressing viruses or envelope portions that comprise a transporter of those viruses.

The mammals immunized with sensitizing antigens are not particularly limited, however are preferably selected considering compatibility with parent cells used for cell fusion. Animals generally used include rodents, lagomorphs, and primates.

Examples of rodents that can be used are mice, rats, and hamsters. As lagomorphs, for example, rabbits can be used. Examples of primates are monkeys. Monkeys that can be used include catarrhines (old-world monkeys) such as cynomolgous monkeys, rhesus monkeys, hamadryas, and chimpanzees.

Animals can be immunized with a sensitizing antigen using known methods. General methods include injecting a sensitizing antigen into a mammal by subcutaneous or intraperitoneal injection. Specifically, a sensitizing antigen is diluted with an appropriate volume of Phosphate-Buffered Saline (PBS) or physiological saline, and if desired, the suspension is mixed with an appropriate volume of a conventional adjuvant, for example, Freund's complete adjuvant. After emulsification, this is applied to the mammals. In addition, after this, the sensitizing antigen that has been mixed with an appropriate volume of Freund's incomplete adjuvant is preferably applied every four to 21 days for several times. When immunizing a

sensitizing antigen, an appropriate carrier can also be used. Thus immunization occurs, and the increased level of the desired antibody in the serum can be confirmed using conventional methods.

Herein, in obtaining the polyclonal antibodies against the transporters of the present invention, the increase in the level of the desired antibody in the serum is confirmed, and blood is then obtained from the mammals sensitized to the antigens. Serum can be separated from this blood using known methods. As polyclonal antibodies, serum comprising polyclonal antibodies can be used. Where necessary, fractions comprising polyclonal antibodies can be isolated from this serum, and this fraction can also be used. For example, fractions that only recognize the transporters of the present invention can be obtained using affinity columns coupled to these transporters. By purifying these fractions using a protein A or protein G column, immunoglobulin G or M can be prepared.

In obtaining monoclonal antibodies, the increased level of the desired antibody is confirmed in the mammals sensitized to the above antigen, immunocytes can be obtained from the mammals, and then subjected to cell fusion. In this case, immunocytes for cell fusion can preferably be splenocytes. As the parent cells to which the above-mentioned immunocytes are bound, mammal myeloma cells are preferable, and more preferable are myeloma cells that have acquired a characteristic for selection of fusion cells using a pharmaceutical agent.

The above-mentioned cell fusion of immunocytes and myeloma cells can be performed according to known methods, for example, the method of Milstein *et al.* (Galfre, G. and Milstein, C., Methods Enzymol. (1981) 73, 3-46).

Hybridomas obtained from the cell fusions can be selected by culturing in a conventional selective culture medium, for example HAT culture medium (medium comprising hypoxanthine, aminopterin, and thymidine). Culture in this HAT culture medium is carried out for a continuous period of usually several days to several weeks, a sufficient time to kill cells other than the target hybridomas (non-fusion cells). Next, conventional limiting dilution methods are carried out, and hybridomas that produce the target antibodies are screened and cloned.

In addition to obtaining the above-mentioned hybridomas by immunizing non-human animals with an antigen, human lymphocytes, for example human lymphocytes infected with EB virus, are sensitized *in vitro* to a virus expressing a transporter of the present invention, or to an envelope portion comprising a transporter of that virus. The sensitized lymphocytes are fused with human-derived myeloma cells that can permanently divide, for example U266. Thus, hybridomas that produce the desired human antibodies that have the activity to bind to the transporters can be obtained (Unexamined Published Japanese Patent Application No. (JP-A) Sho 63-17688).

The obtained hybridomas are transplanted into mice peritoneal cavities, and ascites are recovered from the mice. The monoclonal antibodies thus obtained can be prepared by purification using ammonium sulphate precipitation, protein A or G columns, DEAE ion exchange chromatography, affinity columns to which a transporter of the present invention has been coupled, or the like. In addition to being used for the purification and detection of the transporters of the present invention, the antibodies of the present invention can become candidates for agonists and antagonists of these transporters. Furthermore, these antibodies can also be applied to antibody therapies for diseases involving transporters of the present invention. When using the obtained antibodies for the purpose of application to the human body (antibody therapy), human antibodies and humanized antibodies are preferable due to their low antigenicity.

For example, antibody-producing cells can be obtained by immunizing transgenic animals that comprise a repertoire of human antibody genes, with a virus expressing a transporter that becomes the antigen, or a portion of the viral envelope comprising the transporter. Hybridomas produced by fusing the antibody-producing cells with myeloma cells can be used to obtain human antibodies against the transporter (see International Publication WO92-03918, WO93-2227, WO94-02602, WO94-25585, WO96-33735, and WO96-34096).

In addition to producing antibodies by using hybridomas, immunocytes of antibody-producing sensitized lymphocytes and such that have been immortalized using oncogenes can also be used.

Monoclonal antibodies obtained in this way can also be obtained as recombinant antibodies produced using gene recombination

technologies (for example, see Borrebaeck, C.A.K. and Larrick, J.W., Therapeutic Monoclonal Antibodies, UK, Macmillan Publishers Ltd., 1990). Recombinant antibodies can be produced by cloning DNA that encodes them from immunocytes such as hybridomas and antibody-producing sensitized lymphocytes, incorporating into a suitable vector, and introducing this into a host. The present invention also encompasses such recombinant antibodies.

So long as the antibodies of the present invention bind to the polypeptides of the present invention, they can also be antibody fragments, modified antibodies, etc. For example, an antibody fragment can be an Fab, F(ab')2, Fv, or a single chain Fv (scFv) where Fvs of H chain and L chain are linked by a suitable linker (Huston, J.S. *el al.*, Proc. Natl. Acad. Sci. U.S.A., (1998) 85, 5879-5883). Specifically, the antibody fragments can be produced by treating antibodies with an enzyme such as papain or pepsin. Alternatively, genes encoding these antibody fragments are constructed, inserted into an expression vector, and expressed in appropriate host cells (see for example, Co, M. S. *et al.*, J. Immunol. (1994) 152, 2968-2976; Better, M. and Horwitz, A. H., Methods Enzymol. (1989) 178, 476-496; Pluckthun, A. and Skerra, A., Methods Enzymol. (1989) 178, 497-515; Lamoyi, E., Methods Enzymol. (1986) 121, 652-663 ; Rousseaux, J. *et al.*, Methods Enzymol. (1986) 121, 663-669 ; Bird, R. E. and Walker, B. W., Trends Biotechnol. (1991) 9, 132-137).

Antibodies bound to various molecules such as polyethylene glycols (PEG), can also be used as the modified antibodies. "Antibody" in the present invention also encompasses these modified antibodies. Such a modified antibody can be obtained by chemically modifying obtained antibodies. These methods have already been established in the art.

By using known technologies, the antibodies of the present invention can be obtained as chimeric antibodies comprising non-human antibody-derived variable regions and human antibody-derived constant regions, or alternatively, as humanized antibodies comprising non-human antibody-derived complementarity determining regions (CDRs), human antibody-derived framework regions (FRs), and constant regions.

Antibodies obtained as above can be purified until homogenous.

The separation and purification of antibodies used in the present invention can use conventional separation and purification methods. For example and without limitation, antibodies can be separated and purified by appropriately selecting and combining chromatography columns such as affinity chromatography columns, filters, ultrafiltration, salt precipitation, dialysis, SDS polyacrylamide gel electrophoresis, isoelectric focusing and so on (Antibodies: A Laboratory Manual. Ed Harlow and David Lane, Cold Spring Harbor Laboratory, 1988). The concentration of the above-obtained antibodies can be determined by measuring absorbance, by enzyme-linked immunosorbent assays (ELISA), etc.

Protein A columns, protein G columns, and such can be used as the columns used for affinity chromatography. For example, as the columns using protein A, Hyper D, POROS, Sepharose F.F. (Pharmacia) and so on can be used.

Examples of chromatography other than affinity chromatography include ion exchange chromatography, hydrophobic chromatography, gel filtration, reverse chromatography, and adsorption chromatography (Strategies for Protein Purification and Characterisation: A Laboratory Course Manual. Ed Daniel R, Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996). These chromatographies can be carried out using liquid phase chromatography such as HPLC and FPLC.

Examples of the methods for measuring antigen-binding activities of the antibodies of the present invention include absorbance measurements, enzyme-linked immunosorbent assays (ELISA), enzyme immunoassays (EIA), radioimmunoassay (RIA), and immunofluorescence. When using ELISA, the transporters of the present invention are added to a plate to which the antibodies of the present invention have been solid phased. Next, samples comprising a target antibody, for example the culture supernatant of antibody-producing cells or purified antibodies, are added. Secondary antibodies that recognise the antibody, which is labelled with enzymes such as alkaline phosphatase, are then added and the plate is incubated. After washing, an enzyme substrate such as p-nitrophenol phosphate is added, and antigen-binding activity can be evaluated by measuring absorbance. BIAcore (Pharmacia) can be used to evaluate the activity of the antibodies of the present

invention.

Transporter-binding antibodies can be screened by ELISA using 96-well plates coated with budding baculoviruses. Antibodies against the viral antigens can be removed by ELISA using wild type viruses as the screening antigen. Alternatively, hybridoma culture supernatant and a wild type virus can be reacted, and after antibodies against the viral antigen have been removed, ELISA can be then carried out using a transporter-expressing virus as the screening antigen to acquire transporter-binding antibodies. Function-inhibiting antibodies can also be screened for from the binding antibodies. In other words, a radioactive or fluorescent substrate of the target transporter can be mixed with a solution comprising antibodies, such as hybridoma culture supernatant, and then added to a transporter-expressing virus. The solution comprising antibodies, such as hybridoma culture supernatant, can be preloaded onto the virus prior to adding the substrate. Transport activity in the absence of antibodies is taken as 100, and function-inhibiting antibodies can be screened for using, as an index, decreased activity in the presence of antibodies. Transporter topography at the cellular level can be revealed by binding antibodies to that transporter. In addition, function-inhibiting antibodies can be added to cell cultures or administered to laboratory animals to make a great contribution to the elucidation of the physiological functions of the transporters. Function-inhibiting antibodies or binding antibodies to transporters associated with disease can be applied as pharmaceutical agents.

The present invention can also be used to evaluate the ways in which transporter activity is altered by changes in amino acid sequence due to mutations, polymorphisms such as SNPs, and so on. For example, many SNPs exist in OATP-Cs, and changes in the amino acid sequence due to these SNPs have been reported (J. Biol. Chem., 276 (2001). By using the methods of the present invention to measure the transport activity of each of these OATP-Cs with altered amino acid sequences, the SNPs that influence transport activity can be identified, transporters with high activity can be screened, and so on.

In addition, after mutants have been created by artificial substitution, insertion, deletion, or addition of transporter amino

acid sequences, transporter activity can be measured and transporters with high activity can be screened, or regions that influence transporter activity can be identified. Those skilled in the art can prepare transporters with substituted amino acids by using well-known methods. For example, site-specific mutagenesis and such can be used (Hashimoto-Gotoh, T. *et al.*, Gene, 152, 271-275, (1995); Zoller, M J, and Smith , M., Methods Enzymol, 100, 468-500, (1983); Kramer, W *et al.*, Nucleic Acids Res, 12, 9441-9456, (1984); Kramer, W and Fritz, HJ., Method Enzymol, 154, 350-367, (1987); Kunkel, TA., Proc Natl Acad Sci USA, 82, 488-492, (1985); Kunkel, TA., Methods Enzymol, 85, 2763-2766, (1988)).

Further, when using the present invention, substances transported by a transporter can be used as test substances and measure transporter activity to screen for substances that are easily transported by transporters, or substances that are difficult to transport.

The present invention can also be applied to proteins other than transporters. For example, similar methods for measuring activity, screening and such can be carried out for ion channels such as sodium channels, calcium channels, potassium channels, chloride channels, cation channels, and anion channels. In this case, instead of a transporter, a channel is expressed on the viral envelope, and a substance passed through the channel can be used as a substrate. Channels that can be used in the present invention include those listed in Table 2. Thus, the present invention can be used for proteins that can transport or transmit a substance, such as transporters and ion channels (especially proteins which are expressed on membranes and can be transported or passed in a substrate-specific manner).

In addition to the above transporters and ion channels, the present invention can also be applied to G protein coupled receptors (GPCRs).

Table 2

Symbol	Name	Sequence ID
ACCN1	amiloride-sensitive cation channel 1, neuronal (degenerin)	NM_001094
ACCN2	amiloride-sensitive cation channel 2, neuronal	NM_001095 NM_020039
ACCN3	amiloride-sensitive cation channel 3, testis	NM_004769 NM_020321 NM_020322
AQP1	aquaporin 1 (channel-forming integral protein, 28kD)	NM_000385
ASIC4	putative acid-sensing ion channel	NM_018674
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	NM_000068 NM_023035
CACNA1B	calcium channel, voltage-dependent, L type, alpha 1B subunit	NM_000718
CACNA1C	calcium channel, voltage-dependent, L type, alpha 1C subunit	NM_000719
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	NM_000720
CACNA1E	calcium channel, voltage-dependent, alpha 1E subunit	NM_000721
CACNA1F	calcium channel, voltage-dependent, alpha 1F subunit	NM_005183
CACNA1G	calcium channel, voltage-dependent, alpha 1G subunit	NM_018896
CACNA1H	calcium channel, voltage-dependent, alpha 1H subunit	NM_021098
CACNA1I	calcium channel, voltage-dependent, alpha 1I subunit	NM_021096
CACNA1S	calcium channel, voltage-dependent, L type, alpha 1S subunit	NM_000069
CACNA2D	calcium channel, voltage-dependent, alpha 2/delta subunit 1	NM_000722
CACNA2D	calcium channel, voltage-dependent, alpha 2/delta subunit 2	NM_006030
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	NM_000723
CACNB2	calcium channel, voltage-dependent, beta 2 subunit	NM_000724
CACNB3	calcium channel, voltage-dependent, beta 3 subunit	NM_000725
CACNB4	calcium channel, voltage-dependent, beta 4 subunit	NM_000726
CACNG1	calcium channel, voltage-dependent, gamma subunit 1	NM_000727
CACNG2	calcium channel, voltage-dependent, gamma subunit 2	NM_006078
CACNG3	calcium channel, voltage-dependent, gamma subunit 3	NM_006539
CACNG4	calcium channel, voltage-dependent, gamma subunit 4	NM_014405
CACNG5	calcium channel, voltage-dependent, gamma subunit 5	NM_014404
CACNG6	calcium channel, voltage-dependent, gamma subunit 6	NM_031897
CACNG7	calcium channel, voltage-dependent, gamma subunit 7	NM_031896
CACNG8	calcium channel, voltage-dependent, gamma subunit 8	AF288388
CLCA1	chloride channel, calcium activated, family member 1	NM_001285
CLCA2	chloride channel, calcium activated, family member 2	NM_006536
CLCA3	chloride channel, calcium activated, family member 3	NM_004921
CLCA4	chloride channel, calcium activated, family member 4	NM_012128
CLCN1	chloride channel 1 , skeletal muscle (Thomsen disease, autosomal dominant)	NM_000083

CLCN2	chloride channel 2	NM_004366
CLCN3	chloride channel 3	NM_001829
CLCN4	chloride channel 4	NM_001830
CLCN5	chloride channel 5 (nephrolithiasis 2, X-linked, Dent disease)	NM_000084
CLCN6	chloride channel 6	NM_001286 NM_021735 NM_021736 NM_021737
CLCN7	chloride channel 7	NM_001287
CLCNKA	chloride channel Ka	NM_004070
CLCNKB	chloride channel Kb	NM_000085
CLIC1	chloride intracellular channel 1	NM_001288 NM_001288
CLIC2	chloride intracellular channel 2	NM_001289
CLIC3	chloride intracellular channel 3	NM_004669
CLIC4	chloride intracellular channel 4	NM_013943
CLIC5	chloride intracellular channel 5	NM_016929
CLIC6	chloride intracellular channel 6	BG184920
CLNS1A	chloride channel, nucleotide-sensitive, 1A	NM_001293
CNGA1	cyclic nucleotide gated channel alpha 1	NM_000087
CNGA3	cyclic nucleotide gated channel alpha 3	NM_001298
CNGB1	cyclic nucleotide gated channel beta 1	NM_001297
CNGB3	cyclic nucleotide gated channel beta 3	NM_019098
DKFZP43	potassium channel modulatory factor	NM_020122
ECAC1	epithelial calcium channel 1	NM_019841
ECAC2	epithelial calcium channel 2	AJ243501 AJ243500
HCN2	hyperpolarization activated cyclic nucleotide-gated potassium channel 2	NM_001194
HCN4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4	NM_005477
HSA24339	voltage-gated sodium channel beta-3 subunit (scn3b gene)	NM_018400
HSA27226	calcium channel, voltage-dependent, alpha 2/delta 3 subunit	NM_018398
KCNA1	potassium voltage-gated channel, shaker-related subfamily, member 1 (episodic ataxia with myokymia)	NM_000217
KCNA10	potassium voltage-gated channel, shaker-related subfamily, member 10	NM_005549
KCNA2	potassium voltage-gated channel, shaker-related subfamily, member 2	NM_004974

KCNA3	potassium voltage-gated channel, shaker-related subfamily, member 3	NM_002232
KCNA4	potassium voltage-gated channel, shaker-related subfamily, member 4	NM_002233
KCNA5	potassium voltage-gated channel, shaker-related subfamily, member 5	NM_002234
KCNA6	potassium voltage-gated channel, shaker-related subfamily, member 6	NM_002235
KCNA7	potassium voltage-gated channel, shaker-related subfamily, member 7	NM_031886
KCNAB1	potassium voltage-gated channel, shaker-related subfamily, beta member 1	NM_003471
KCNAB2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	NM_003636
KCNAB3	potassium voltage-gated channel, shaker-related subfamily, beta member 3	NM_004732
KCNB1	potassium voltage-gated channel, Shab-related subfamily, member 1	NM_004975
KCNB2	potassium voltage-gated channel, Shab-related subfamily, member 2	NM_004770
KCNC1	potassium voltage-gated channel, Shaw-related subfamily, member 1	NM_004976
KCNC3	potassium voltage-gated channel, Shaw-related subfamily, member 3	NM_004977
KCNC4	potassium voltage-gated channel, Shaw-related subfamily, member 4	NM_004978
KCND1	potassium voltage-gated channel, Shal-related subfamily, member 1	NM_004979
KCND2	potassium voltage-gated channel, Shal-related subfamily, member 2	NM_012281
KCND3	potassium voltage-gated channel, Shal-related subfamily, member 3	NM_004980
KCNE1	potassium voltage-gated channel, Isk-related family, member 1	NM_000219
KCNE1L	potassium voltage-gated channel, Isk-related family, member 1-like	NM_012282
KCNE2	potassium voltage-gated channel, Isk-related family, member 2	NM_005136
KCNE3	potassium voltage-gated channel, Isk-related family, member 3	NM_005472
KCNF1	potassium voltage-gated channel, subfamily F, member 1	NM_002236

KCNG1	potassium voltage-gated channel, subfamily G, member 1	NM_002237
KCNG2	potassium voltage-gated channel, subfamily G, member 2	NM_012283
KCNH1	potassium voltage-gated channel, subfamily H (eag-related), member 1	NM_002238
KCNH2	potassium voltage-gated channel, subfamily H (eag-related), member 2	NM_000238
KCNH3	potassium voltage-gated channel, subfamily H (eag-related), member 3	AB033108
KCNH4	potassium voltage-gated channel, subfamily H (eag-related), member 4	NM_012285
KCNH5	potassium voltage-gated channel, subfamily H (eag-related), member 5	U69185
KCNIP1	Kv channel-interacting protein 1	NM_014592
KCNIP2	Kv channel-interacting protein 2	NM_014591
KCNJ1	potassium inwardly-rectifying channel, subfamily J, member 1	NM_000220
KCNJ10	potassium inwardly-rectifying channel, subfamily J, member 10	NM_002241
KCNJ11	potassium inwardly-rectifying channel, subfamily J, member 11	NM_000525
KCNJ12	potassium inwardly-rectifying channel, subfamily J, member 12	NM_021012
KCNJ13	potassium inwardly-rectifying channel,subfamily J, member 13	AJ007557
KCNJ14	potassium inwardly-rectifying channel,subfamily J, member 14	NM_013348
KCNJ15	potassium inwardly-rectifying channel, subfamily J, member 15	NM_002243
Symbol	Name	Sequence ID
KCNJ16	potassium inwardly-rectifying channel, subfamily J, member 16	NM_018658
KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	NM_000891
KCNJ3	potassium inwardly-rectifying channel, subfamily J, member 3	NM_002239
KCNJ4	potassium inwardly-rectifying channel, subfamily J, member 4	NM_004981
KCNJ5	potassium inwardly-rectifying channel, subfamily J, member 5	NM_000890
KCNJ6	potassium inwardly-rectifying channel, subfamily J, member 6	NM_002240
KCNJ8	potassium inwardly-rectifying channel, subfamily J, member 8	NM_004982
KCNJ9	potassium inwardly-rectifying channel, subfamily J, member 9	NM_004983
KCNJN1	potassium inwardly-rectifying channel, subfamily J, inhibitor 1	NM_002244
KCNK1	potassium channel, subfamily K, member 1 (TWIK-1)	NM_002245
KCNK10	potassium channel, subfamily K, member 10	NM_021161
KCNK12	potassium channel, subfamily K, member 12	NM_022055
KCNK13	potassium channel, subfamily K, member 13	NM_022054
KCNK2	potassium channel, subfamily K, member 2 (TREK-1)	AF004711
KCNK3	potassium channel, subfamily K, member 3 (TASK-1)	NM_002246
KCNK4	potassium inwardly-rectifying channel, subfamily K, member 4	NM_016611
KCNK5	potassium channel, subfamily K, member 5 (TASK-2)	NM_003740
KCNK6	potassium channel, subfamily K, member 6 (TWIK-2)	NM_004823

KCNK7	potassium channel, subfamily K, member 7	NM_005714
KCNK9	potassium channel, subfamily K, member 9 (TASK-3)	NM_016601
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1	NM_002247
KCNMB1	potassium large conductance calcium-activated channel, subfamily M, beta member 1	NM_004137
KCNMB2	potassium large conductance calcium-activated channel, subfamily M, beta member 2	NM_005832
KCNMB3	potassium large conductance calcium-activated channel, subfamily M beta member 3	NM_014407
KCNMB3L	potassium large conductance calcium-activated channel, subfamily M, beta member 3-like	NM_014406
KCNMB4	potassium large conductance calcium-activated channel, subfamily M, beta member 4	NM_014505
KCNN1	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1	NM_002248
KCNN2	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2	NM_021614
KCNN3	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 3	NM_002249
KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	NM_002250
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member	NM_000218
KCNQ2	potassium voltage-gated channel, KQT-like subfamily, member	NM_004518
KCNQ3	potassium voltage-gated channel, KQT-like subfamily, member	NM_004519
KCNQ4	potassium voltage-gated channel, KQT-like subfamily, member	NM_004700
KCNQ5	potassium voltage-gated channel, KQT-like subfamily, member	NM_019842
KCNS1	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1	NM_002251
KCNS2	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 2	AB032970
KCNS3	potassium voltage-gated channel, delayed-rectifier, subfamily S, member 3	NM_002252
KIAA0439	homolog of yeast ubiquitin-protein ligase Rsp5; potential epithelial sodium channel regulator	AB007899
KIAA1169	two-pore channel 1, homolog	NM_017901
KV8.1	neuronal potassium channel alpha subunit	NM_014379
LOC64181	two pore potassium channel KT3.3	NM_022358
OTRPC4	vanilloid receptor-related osmotically activated channel; OTRPC4 protein	NM_021625

P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1	NM_002558
P2RX2	purinergic receptor P2X, ligand-gated ion channel, 2	NM_012226 NM_016318
P2RX3	purinergic receptor P2X, ligand-gated ion channel, 3	NM_002559
P2RX4	purinergic receptor P2X, ligand-gated ion channel, 4	NM_002560
P2RX5	purinergic receptor P2X, ligand-gated ion channel, 5	NM_002561
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7	NM_002562
SCN10A	sodium channel, voltage-gated, type X, alpha polypeptide	NM_006514
SCN11A	sodium channel, voltage-gated, type XI, alpha polypeptide	AF188679
SCN12A	sodium channel, voltage-gated, type XII, alpha polypeptide	NM_014139
SCN1A	sodium channel, voltage-gated, type I, alpha polypeptide	AF225985
SCN1B	sodium channel, voltage-gated, type I, beta polypeptide	NM_001037
SCN2A2	sodium channel, voltage-gated, type II, alpha 2 polypeptide	NM_021007
SCN2B	sodium channel, voltage-gated, type II, beta polypeptide	NM_004588
SCN3A	sodium channel, voltage-gated, type III, alpha polypeptide	AF225987
SCN4A	sodium channel, voltage-gated, type IV, alpha polypeptide	NM_000334
SCN5A	sodium channel, voltage-gated, type V, alpha polypeptide (long (electrocardiographic) QT syndrome 3)	NM_000335
SCN6A	sodium channel, voltage-gated, type VI, alpha polypeptide	NM_002976
SCN8A	sodium channel, voltage gated, type VIII, alpha polypeptide	NM_014191
SCN9A	sodium channel, voltage-gated, type IX, alpha polypeptide	NM_002977
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	NM_001038
SCNN1B	sodium channel, nonvoltage-gated 1, beta (Liddle syndrome)	NM_000336
SCNN1D	sodium channel, nonvoltage-gated 1, delta	NM_002978
SCNN1G	sodium channel, nonvoltage-gated 1, gamma	NM_001039
TALK-1	pancreatic 2P domain potassium channel TALK-1	NM_032115
TASK-4	potassium channel TASK-4; potassium channel TALK-2	NM_031460
TRPC1	transient receptor potential channel 1	NM_003304
TRPC2	transient receptor potential channel 2	X89067
TRPC3	transient receptor potential channel 3	NM_003305
TRPC4	transient receptor potential channel 4	NM_016179
TRPC5	transient receptor potential channel 5	NM_012471
TRPC6	transient receptor potential channel 6	NM_004621
TRPC7	transient receptor potential channel 7	NM_003307
VDAC1	voltage-dependent anion channel 1	NM_003374
VDAC1P	voltage-dependent anion channel 1 pseudogene	AJ002428
VDAC2	voltage-dependent anion channel 2	NM_003375
VDAC3	voltage-dependent anion channel 3	NM_005662
trp7	putative capacitative calcium channel	NM_020389

Brief Description of the Drawings

Fig. 1 is a graph showing PepT1 activity in PepT1-expressing viruses. The PepT1 activity on the viral envelope was measured as the amount of ^{14}C glycylsarcosine uptake by the viruses. "Wild type" shows the amount taken up by the wild type virus. "His-PepT1" shows the amount taken up by a PepT1-expressing virus with a His-tag added to the N-terminal.

Fig. 2 is a graph showing PepT2 activity in PepT2-expressing viruses. The PepT2 activity on the viral envelope was measured as the amount of ^{3}H glycylsarcosine uptake by viruses. "Wild type" shows the amount taken up by the wild type virus. "His-PepT2" shows the amount taken up by a PepT2-expressing virus with a His-tag added to the N-terminal.

Fig. 3 is a graph showing OATP-C activity in OATP-C-expressing viruses. The OATP-C activity on the viral envelope was measured as the amount of ^{3}H estrone sulphate conjugate taken up by viruses. "Wild type" shows the amount taken up by the wild type virus. "OATP-C WT" shows the amount taken up by a wild-type OATP-C-expressing virus. "OATP-C N130D" shows the amount taken up by a N130D mutant OATP-C-expressing virus. "OATP-C V147A" shows the amount taken up by a V147A mutant OATP-C-expressing virus. Each of these OATP-Cs comprises a His-tag added to the N-terminal.

Fig. 4 is a graph showing the results of detecting inhibition of the PepT1 activity in PepT1-expressing viruses by an anti-human PepT1 monoclonal antibody. The PepT1 activity on the viral envelope was measured as the amount of ^{14}C glycylsarcosine taken up by the viruses. Data is shown as the mean +/- SD (n=3-4).

Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using Examples, however, it is not to be construed as being limited to thereto.

[Example 1]

1. Preparation of PepT1-expressing budding baculoviruses

A full-length PepT1 gene was isolated from a human kidney library using PCR. By inserting the full-length human PepT1 gene into pBlueBacHis2A (Invitrogen), the pBlueBacHis-PepT1 transfer vector

was constructed. A Bac-N-Blue transfection kit (Invitrogen) was then used to introduce this transfer vector into Sf9 cells, along with Bac-N-Blue DNA. Thus, a recombinant virus for the expression of human PepT1 was constructed. Specifically, 4 μg of pBlueBacHis-PepT1 was added to Bac-N-Blue DNA, and then 1 mL of Grace's medium (GIBCO) and 20 μL of cell FECTIN reagent was added. This was mixed, incubated for 15 minutes at room temperature, and then added drop-by-drop to $2x\ 10^6$ Sf9 cells washed once with the Grace's medium. After incubating for four hours at room temperature, 2 mL of complete medium (Grace's medium which comprises 10% fetal bovine serum (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (GIBCO-BRL)) was added and cultured at 27°C. Recombinant viruses for expressing human PepT1, which were constructed by homologous recombination, were cloned twice according to the instructions attached to the kit. A virus stock of the recombinant viruses was thus obtained.

Construction of budding-type viruses that express human PepT1 was carried out as follows. Specifically, 500 mL of Sf9 cells ($2x\ 10^6$/mL) were infected with the recombinant viruses prepared as above, so as to achieve MOI=5. After culturing at 27°C for three days, the culture supernatant was centrifuged for 15 minutes at 800x g, and the cells and cell debris were removed. The supernatant recovered by centrifugation was centrifuged at 45,000x g for 30 minutes, and the precipitate was then suspended in PBS. The cellular components were removed by centrifuging for another 15 minutes at 800x g. The supernatant was again centrifuged at 45,000x g for 30 minutes, and the precipitate was again suspended in PBS. This suspension was the budding virus fraction. Expression of PepT1 in the virus and on the Sf-9 cell membrane was confirmed by Western analysis using anti-His antibodies. In addition, protein concentration was measured using Dc Protein Assay kit (Bio-Rad), with BSA as the standard.

2. PepT1 functional analysis

^{14}C glycylsarcosine was diluted with HBSS (pH6.0) to a final concentration of 50 μM, and used as a substrate solution. 40 μL of viral solution (100 μg protein) was preincubated at 37°C for 30 minutes. 160 μL of substrate solution that had been preheated to 37°C was added, and the reaction was started. After one minute, 1 mL of ice-cold HBSS

(pH 7.4)(hereinbelow also called "quenching solution") was added, and the reaction was stopped. The virus-comprising reaction solution was immediately vacuum filtered using a mixed cellulose membrane filter, and washed twice with 5 mL of the quenching solution. The membrane filter was transferred to a liquid scintillation vial, 5 mL of clear-zol I was added, and the filter was dissolved. After the dissolving, a liquid scintillation counter was used to measure radioactivity on the filter. Non-specific adsorption to the filter was measured in the same way for systems where the quenching solution was added before adding the substrate solution to the viral solution, and values thus obtained were subtracted from the counts for each experiment.

The PepT1 activity of the PepT1-expressing virus with a His-tag added at its N-terminal is shown in Fig. 1. A PepT1 activity ratio of about seven times that of the wild type virus not expressing PepT1 was detected.

[Example 2]

1. Preparation of PepT2-expressing budding baculoviruses

The full-length PepT2 gene was isolated from a human kidney library. PCR was used to integrate the gene encoding the full-length human PepT2 into pBlueBacHis2A (Invitrogen), and a full-length PepT2 transfer vector (pBlueBac) was constructed. This vector was introduced into Sf-9 cells along with the viral DNA. After cloning the recombinant virus constructed by homologous recombination, a stock with a high recombinant virus activity was constructed. Sf-9 cells were infected with the stock virus, and after culturing for a certain period, PepT2 was expressed in the virus and on the membrane of Sf-P cells. PepT2 expression in the virus and on the membrane of the Sf-9 cells was confirmed by Western analysis using anti-His antibodies. More specifically, except for using the PepT2 gene, operations were carried out according to the methods described in Example 1.

2. PepT2 functional analysis

^{3}H glycylsarcosine was diluted with HBSS (pH6.0) to a final concentration of 0.8 μM, and used as a substrate solution. 40 μL of

viral solution (100 μg protein) was preincubated at 37°C for 30 minutes. 160 μL of the substrate solution preheated to 37°C was added to commence the reaction. After one minute, 1 mL of the quenching solution was added, and the reaction was stopped. The virus-comprising reaction solution was immediately vacuum filtered using a mixed cellulose membrane filter, and washed twice with 5 mL of the quenching solution. The membrane filter was transferred to a liquid scintillation vial, 5 mL of clear-sol I was added, and the filter was dissolved. After the dissolving, a liquid scintillation counter was used to measure radioactivity on the filter. The quenching solution was added before adding the substrate solution to the viral solution, and similar manipulations were performed. Non-specific adsorption to the filter was measured and the obtained value was subtracted from the counts for each experiment.

The PepT2 activity of the PepT2-expressing virus with a His-tag added to its N-terminal is shown in Fig. 2. A PepT2 activity ratio of about nine times that of the wild type virus not expressing PepT2 was detected.

[Example 3]

1. Preparation of OATP-C expressing baculoviruses

cDNA encoding wild type human OATP-C (OATP-C WT) was cloned as follows. Specifically, adult human liver-derived cDNA was used as a template, and the OATP-C WT cDNA was divided into two fragments and amplified using PCR with the following primer combinations:

5′ side

OAHC17 primer.5′ gat ggt acc aaa ctg agc atc aac aac aaa aac 3′ (SEQ ID NO: 1)

OAHC18 primer: 5′ gat ggt acc cat cga gaa tca gta gga gtt atc 3′ (SEQ ID NO: 2)

3′side

OAHC21 primer: 5′ gat ggt acc tac cct ggg atc tct gtt ttc taa 3′ (SEQ ID NO: 3)

OAHC22 primer: 5′ gat ggt acc gtt tgg aaa cac aga agc aga agt 3′ (SEQ ID NO: 4)

Each of these fragments were subcloned to pT7Blue-T vector (Novagen), and clones without PCR errors were selected. Both were

linked at the BglII site which exists in an overlapping region, and then cleaved at the KpnI site that exists on both ends. After incorporation at the KpnI site of pcDNA3 vector (Invitrogen), pcDNA3/OATP-C WT was obtained.

Next, with pcDNA3/OATP-C WT as a template, *in vitro* mutageneis using GeneEditor™ (Promega) was used to prepare cDNAs coding for OATP-C N130D in which the 130th asparagine was mutated to aspartic acid, and OATP-C V174A in which the 174th valine was mutated to alanine. The primers used for mutagenesis were as follows:

Primer for OATP-C N130D: 5′ gaa act aat atc gat tca tca gaa aat 3′ (SEQ ID NO: 5)

Primer for OATP-C V174A: 5′ atg tgg ata tat gcg ttc atg ggt aat 3′ (SEQ ID NO: 6)

The primers for use in mutagenesis and the selection primers included in kits (for bottom strand use) were both annealed to the template plasmid DNA, which had been made into a single strand. Thus, a new DNA strand was constructed. This was introduced into *E. coli*, and GeneEditor™ antibiotic-resistant clones were obtained. These clones were sequenced and clones containing mutations were thus selected (pcDNA3/OATP-C N130D and pcDNA3/OATP-C V174A).

Next, using pcDNA3/OATP-C WT, pcDNA3/OATP-C N130D and pcDNA3/OATP-C V174A as respective templates, PCR was carried out using the primers below, thus amplifying the respective cDNAs with SalI sites on each end.

C45 primer: 5′ gat gtc gac tta aca atg tgt ttc act 3' (SEQ ID NO 7)

C58 primer: 5′ gat gtc gac tat gga cca aaa tca aca t 3' (SEQ ID NO: 8)

These were digested with SalI, and then inserted into the SalI site of the pBlueBac His2A vector (Invitrogen). Thus transfer vectors encoding each OATP-C protein with a His-tag attached at the N-terminal were constructed (pBlueBac His2A/OATP-C WT, pBlueBac His2A/OATP-C N130D, pBlueBac His2A/OATP-C V174A).

Using Bac-N-Blue transfection kit (Invitrogen), these vectors were introduced into Sf-9 cells along with viral DNA. After five to eight days, plaque assays were used to clone the recombinant viruses in the culture supernatant. The viruses were then amplified, and a stock of highly active recombinant viruses was prepared. Sf-9 cells

were infected with the stock viruses at MOI=1. After four days, recombinant viruses were recovered from the culture supernatant. OATP-C expression on the viral envelope was confirmed by Western analysis using anti-His antibodies.

2. OATP-C functional analysis.

^{3}H estrone sulphate conjugate was diluted with HBSS (pH7.4) to a final concentration of 10 nM, and used as a substrate solution. 20 μL of viral solution (50 μg protein) was preincubated at 37°C for 30 minutes. 180 μL of the substrate solution preheated to 37°C was added, and the reaction was started. After one minute, 1 mL of ice-cold HBSS (pH7.4) (hereinafter referred to as "quenching solution") was added, and the reaction was stopped. The virus-comprising reaction solution was immediately vacuum filtered using a mixed cellulose membrane filter, and washed twice with 5 mL of the quenching solution. The membrane filter was transferred to a liquid scintillation vial, 5 mL of clear-sol I was added, and the filter was dissolved. After the dissolving, a liquid scintillation counter was used to measure radioactivity on the filter. To measure non-specific adsorption to the filter, the reaction quenching solution was added before adding the substrate solution and similar manipulations were performed. The obtained value was subtracted from the counts for each experiment.

The activity of ^{3}H estrone sulphate conjugate uptake is shown in Fig. 3 for three types of OATP-C-expressing viruses with His-tags added to their N-terminals. The detected ^{3}H estrone sulphate conjugate uptake activity ratios for wild type OATP-C, N130D, and V174A were respectively 57, 41, and 36 times that of a wild type virus not expressing OATP-C. In addition, virus-derived endogenous OATP-C activity was hardly detected in experiments on the uptake in wild type viruses. Thus, it was revealed that budding baculovirus expression systems are systems with extremely low background levels. Furthermore, since OATP-C mutants (N130D, V174A) can be functionally expressed on viral envelopes, changes in substrate specificity due to SNPs can also be determined, making also applications to tailor-made therapy possible.

[Example 4] Search for PepT1 function inhibiting antibodies

^{14}C glycylsarcosine was diluted with HBSS (pH 6.0) to a final concentration of 50 μM, and used as a substrate solution. In addition, a mouse monoclonal antibody recognising the extracellular region of human PepT1 was diluted with PBS to a final concentration of 200 μg/mL, and used as an antibody solution. 20 μL (50 μg protein) of solution of budding baculoviruses expressing PepT1 with a His-tag added at the N-terminal was mixed with 20 μL of the antibody solution and incubated for one hour at 37°C. 160 μL of substrate solution preheated to 37°C was added, and the reaction was started. After one minute, 1 mL of ice-cold HBSS (pH 7.4) (below also called "quenching solution") was added, and the reaction was stopped. The virus-comprising reaction solution was immediately vacuum filtered using a mixed cellulose membrane filter, and washed twice with 5 mL of the quenching solution. The membrane filter was transferred to a liquid scintillation vial, 5 mL of clear-sol I was added, and the filter was dissolved. After the dissolving, a liquid scintillation counter was used to measure radioactivity on the filter. Non-specific adsorption to the filter was measured by adding the reaction quenching solution before adding the substrate solution to the viral solution, and performing similar manipulations. The obtained value was subtracted from the counts for each experiment.

The PepT1 activity inhibition by the anti-human PepT1 monoclonal antibodies is shown in Fig. 4. PepT1 activity in the absence of the antibodies was taken as the control and expressed as 100. Of the three types of anti-human PepT1 monoclonal antibodies, clone 119 inhibited PepT1 activity by about 20%, and clone 253 by about 10%, compared to the control. This PepT1 activity inhibition was statistically significant (Student t-test). Thus, budding baculovirus expression systems will be useful in the search for substrates that inhibit or promote transporter activity.

Industrial applicability

The present invention provides viruses that express transporters having transporter activity, and by using these viruses, transporter activity can be measured with a high sensitivity and less background level than in the past. Thus, it is expected that by

employing the methods of the present invention, identification of transport substrates and driving force of transporters, and functional analysis such as kinetic analysis can be carried out more easily and accurately than before. In addition, by using such viruses, substances that inhibit or promote the transport activities of transporters expressed on the viral envelopes can be screened. Since transporters have also been reported to be involved in the transport of drugs into cells, substances that inhibit or promote the activities of transporters associated with diseases can become candidates for new pharmaceutical agents. Furthermore, by using the methods of the present invention for analysis of SNPs in transporter-encoding genes, functional changes due to transporter SNPs can be measured over a more extensive range of substrates. Application to tailor-made therapies is also possible since response to a drug can be analyzed for each individual.